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## **Shigella spp. and entero-invasive Escherichia coli**

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An abstract graphic on the left side of the page, consisting of a dense, overlapping network of thin red lines that form a spherical shape, resembling a wireframe or a complex geometric pattern.

## Chapter 1

### General introduction

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## Introduction

### The pathogen and the disease

Hippocrates (460-370 B.C.) was the first to describe a disease characterized by the passing of frequent bloody and mucoid stools that came along with straining and tenesmus. He named it dysentery, which translates as “bowel trouble” from ancient Greek [1]. Since then, many more cases were described, often in the form of large devastating epidemics accompanied with displaced human population due to wars or natural disasters [2-4]. Until the end of the 19<sup>th</sup> century, when the pathogens that cause dysentery were first described, bacillary dysentery could not be distinguished from amoebic dysentery [1, 2]. After the discovery of *Entamoeba histolytica* in 1875, Kiyoshi Shiga discovered the cause of bacillary dysentery in Japan in 1898 [5]. During an epidemic, he cultured the bacterium ‘*Bacillus dysenteriae*’, nowadays referred to as *Shigella dysenteriae*, from fecal samples of dysentery patients. Additionally, he discovered that these isolates were able to agglutinate with the serum of the infected patients [4, 5]. Later, other related organisms were discovered with slightly different metabolic activities and antigenic properties. In 1899, Simon Flexner described the Flexner bacillus that we now call *Shigella flexneri* from patients from the Philippines. In 1915, Carl Sonne first described a group III dysentery causing bacterium ‘*B. dysenteriae* Sonne’, now called *Shigella sonnei*. It caused most cases of dysentery in Denmark [5]. Finally, Major Boyd of the British Army Medical Corps described isolation of *Shigella boydii* from Indian patients in 1929. He was also the founder of the disposition of *Shigella* into different antigenic groups, a classification that is used until this day [4].

In 1940, gram-negative bacteria were isolated from patients with dysentery in the Netherlands, yet these bacteria showed metabolic activities that resemble *Escherichia coli* more than the described properties for the genus *Shigella* [6]. These *E. coli* isolates had O-type O28ac, and from then until 1977 other isolates resembling *E. coli*, but capable to cause dysentery were described, and were assigned as the pathotype entero-invasive *E. coli* (EIEC) [7, 8]. More descriptions of EIEC associated O-types followed from the 1970s up to now [9-12].

Globally, diarrhea in general was recently estimated to cause annually 1.3 million deaths and 71.6 million disability-adjusted life-years (DALYs) [13]. It was estimated that in 2016 *Shigella spp.* accounted for approximately 210,000 of those deaths amongst all age groups, as second leading accountable pathogen after Rotavirus, with 63,000 deaths amongst children under five [14]. From another study, that assessed the etiologies of childhood moderate-to-severe diarrheal cases in Africa and South Asia, it followed that *Shigella spp.* were the fourth leading cause for diarrhea under 12 months of age, and the 1<sup>st</sup> accountable pathogen for children from 1 year of age and above [15, 16]. Although mortality due to *Shigella spp.* is primarily present in resource-restricted areas, shigellosis also pose a

substantial burden on resource-rich areas such as the Netherlands, due to 196 disability-adjusted life years/year, the use of healthcare facilities, and employment of disease control measures [17, 18].

The burden of diarrhea caused by EIEC was seldomly investigated. However, one multisite case-control study performed in South America, Africa and Asia, found that EIEC was one of the pathogens amongst children that was associated with mild to severe diarrheal complaints accompanied by blood in stool [19].

*Shigella spp.* and EIEC can cause diarrhea, ranging from mild diarrheal episodes until complete dysentery [20-22]. Its clinical picture starts generally with fever, headache, loss of appetite, malaise and vomiting followed by watery diarrhea. In mild infections in healthy persons, symptoms will usually diminish within a few days to a week without treatment. However, for some patients, the disease will progress into dysentery, characterized by bloody and mucoid diarrhea with frequent passage of redcurrant jelly resembling stools, accompanied by abdominal pain, abdominal cramps and tenesmus [2, 20]. Complications resulting from infections with *Shigella spp.* are uncommon, but do occur and consist of intestinal complications as rectal prolapse or toxic megacolon, or extra-intestinal complications, as seizures or sepsis. The latter complications are predominantly observed in children and HIV+ patients [20]. Following infection with *Shigella spp.*, irritable bowel syndrome, reactive arthritis and hemolytic uremic syndrome (HUS) can develop [20].

### Taxonomy and evolution of *Shigella spp.* and *Escherichia coli*

Bacterial taxonomy is the science that issues the classification, nomenclature and identification of bacterial taxa, for practicality and manageability in the scientific world. First, classification is the disposition of new discovered taxa in the established framework of the bacterial taxonomy [23]. Second, with nomenclature, these taxa are named and a type strain is assigned according to rules described in the International Code of Nomenclature of Prokaryotes [24]. According to rule 23 of this code, newly proposed bacterial classifications and their corresponding names can only be conserved or rejected through an official request for opinion issued by the Judicial Commission [24]. Finally, identification is the assignment of unknown isolates to earlier classified and named taxa. In the early days, bacterial taxonomists classified new taxa by studying the morphological, metabolic and antigenic characteristics of newly described bacteria. From the 1970s, standards for classification were described as polyphasic taxonomy, which consists of the application of a range of techniques to characterize phenotypical, chemotaxic and genomic traits of the bacteria. In this polyphasic taxonomy, 16S rRNA gene analysis and DNA-DNA hybridization experiments were considered as the gold standard of bacterial classification and nomenclature [25, 26]. Since the last two decades, methods for classification and nomenclature are shifting towards genomic descriptions [27-30].



Although the genus name “*Shigella*” was first mentioned in the 3<sup>rd</sup> edition of Bergey’s manual of determinative bacteriology from 1930 [31], the Judicial Commission issued the official classification of *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* in 1954 as species within a conserved separate genus, named *Shigella* [24]. Because other techniques were not yet available at the time, the classification was based on morphological, metabolic and antigenic properties in particular.

Since the discovery of *S. dysenteriae* by Dr. Shiga in 1898, the resemblance to *E. coli* was recognized. However, the extent of this resemblance was not proved until after the official assignment of *Shigella spp.* in 1954 when relatively modern techniques became available. Since that time, it was demonstrated that the actual genetic relatedness of *Shigella spp.* and *E. coli* was very high and their evolution was intertwined. Even before the structure of DNA was unraveled, in 1957, Salvador Luria and Jeanne Burrous performed phage transduction experiments with different *Enterobacteriaceae* and discovered that *Shigella* can “mate” with *E. coli*, creating recombinant strains that they called “monstrosities from the standpoint of traditional bacterial classification” [32]. They were ahead of their time, by also hypothesizing that this kind of recombination could occur in nature, for instance in the human gut, and that it can play a role in the evolution of *Enterobacteriaceae* [32]. When in the 1970s polyphasic taxonomy was proposed, Don Brenner and his group showed by multiple DNA-DNA hybridization experiments the close genetic relationship of *Shigella* with *E. coli* [33, 34]. In fact, they detected hybridization percentages above the 70% species boundary, indicating they should belong to the same species. In 1998, Henrik Christensen *et al.* showed that *Shigella* and *E. coli* form a tight complex based on their 16S rRNA sequences, close to the genus *Salmonella* [35]. Pioneers in whole genome sequencing showed that *Shigella spp.* and *E. coli* genomes share a common backbone, which is interspersed with many insertion (IS) elements in *Shigella spp.* [36, 37]. These mobile elements contribute to a dynamic genome that promotes the pathogenicity of *Shigella spp.* by easy gain and loss of genes enabling adaptation to different circumstances in- and outside the human host [36, 38, 39]. Although it was demonstrated that classification of *Shigella* as separate genus from *E. coli* does not reflect the actual genetic relatedness, it is still maintained due to historical, practical and clinical reasons [40, 41].

To assess the relation of *Shigella spp.* with the pathotype EIEC in particular, several experiments were performed that sequenced multiple housekeeping genes of *Shigella* and EIEC [42-44]. These studies indicated that *Shigella spp.* has risen on different occasions from multiple different ancestors within the group of *E. coli*. *Shigella*-EIEC forms one single pathovar, in which the same species or serotypes are not necessary phylogenetically related to each other [43]. Additionally, they estimated using molecular clock rates that the major lineages of *Shigella spp.* are derived from a common ancestor 35,000–270,000 years ago [42]. This evolution occurred relatively recently when one takes into account that a major non-

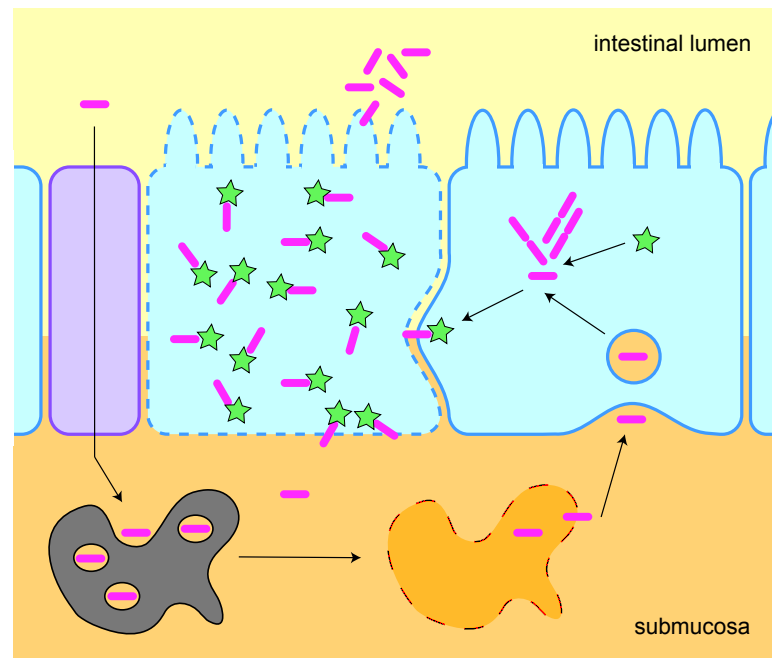
pathogenic *E. coli* cluster diverged from other bacteria 8–22 million years ago. The estimated time of derivation of *Shigella spp.* coincides with the Paleolithic expansion and the rise of early man. This is probably no coincidence as pathogenesis of *Shigella spp.* is based on surviving in the intestinal epithelial cells of humans only, representing a perfect host-adaptation [42, 44, 45]. Sequence variations in the lineages of *Shigella spp.* and EIEC have indicated that EIEC evolved from *E. coli* ancestors later than *Shigella spp.* Based on this later derivation of EIEC, two hypotheses about EIEC in relation to *Shigella spp.* were posed by Lan *et al.* [43]. The first hypothesis is that EIEC is an ancestral form that in time will develop into ‘real’ *Shigella*. The second hypothesis is that EIEC is a different group of organisms that is adapted to human hosts like *Shigella spp.*, but is better equipped to survive outside the host [43]. The major event that probably initiated the divergence of *Shigella spp.* and EIEC from other *E. coli* is the acquisition of the invasion plasmid (pINV) that encodes virulence genes necessary for the invasive phenotype [46].

The proposed common evolutionary history and the presence of a genetically tighter group of *Shigella spp.* and EIEC within the species *E. coli* was later confirmed with multiple experiments based on whole genome sequencing [47-50].

### Virulence

*Shigella spp.* and EIEC both cause invasive enteral infections by invading the intestinal epithelial cells followed by intracellular multiplication and spread to adjacent cells [46, 51-54]. First, the bacteria in the intestinal lumen invade the submucosal side of the human colon by transcytosis through microfold cells, as depicted in Figure 1 [54]. Once they access the submucosa, *Shigella spp.* or EIEC are engulfed by macrophages. The bacteria induce death of the macrophages, and are released in the submucosa from where they invade epithelial cells by endocytosis (Figure 1). Once inside the endocytic vacuoles, the bacteria escape rapidly into the cytoplasm of the host cell, where they replicate (Figure 1) [53, 54]. For intra and extracellular spread, *Shigella spp.* and EIEC exploit the actin machinery present in the epithelial cells [53]. To get access to adjacent host cells, the bacteria cause protrusions close to intercellular junctions, which are endocytosed by pathways that are part of the inflammatory response of the host. This results in local lysis of the membranes of the adjacent epithelial cells (Figure 1). If multiple epithelial cells are disrupted, the submucosal side is freely accessible for more bacteria to cause enhanced infection (Figure 1) [53]. Thus, *Shigella spp.* and EIEC need and exploit the inflammatory response of the human host for their effective and perfectly human adapted methods of invasion. However, in the end, the host immune response system will be able to clear the infection [53].

As mentioned, responsible for the invasive phenotype is the acquisition of the pINV, a large single-copy plasmid of 180–230, which is harbored by all virulent *Shigella spp.* and EIEC [55-58]. The pINVs of different species form a closely related family and share a common



**Figure 1** Schematic overview of the infective mechanism of *Shigella* spp. and EIEC.

Pink rods = *Shigella* spp./EIEC; Purple cell = microfold cell; gray cell = macrophage; blue cells = epithelial cells; green star = actin

origin outside *E. coli*. Although the pINVs evolved independently, the virulence genes are highly conserved and proved to be functionally interchangeable between different serotypes [55, 58, 59]. The genes most essential for invasion of *Shigella* spp. and EIEC are the *mxi* and *spa* genes that encode the type III secretion system (T3SS), a temperature regulated, needle-shaped delivery system. It delivers the T3SS effectors, also encoded on the pINV, which are secreted into the host cells upon contact [22, 53]. Because the maintenance of the T3SS region has high fitness costs, the bacteria can excise this region while being outside the human host, using at least four different pathways, both reversible and irreversible [39].

To display this invasive mechanism, *Shigella* spp. and EIEC not only need expression from virulence genes encoded by the pINV, but also from virulence genes present on the chromosome [46, 53, 58, 60]. The chromosomes of *Shigella* spp. and EIEC have adapted to the acquisition of the invasion plasmid by pathoadaptation, which can occur by multiple different events, such as bringing newly acquired virulence genes under control of an already present regulator, by point mutations within genes, by the suppression or expression of genes, or by deletion of anti-virulence genes [60, 61]. The virulence genes present on the chromosome of *Shigella* spp. or EIEC are predominantly located in large pathogenicity islands (PAIs), called SHI-1, SHI-2 or SHI-3 [53].

Additionally, multiple toxins produced by *Shigella* spp. and EIEC were described. The first described and most notorious toxin in *Shigella* spp. is the shiga-toxin encoded by the *stx* gene. This toxin activates platelets, which adhere to the endothelium and obstruct blood vessels leading to microangiopathic hemolysis causing hemorrhagic colitis and hemolytic uremic syndrome (HUS) [20, 62]. Historically, this toxin was only described in *S. dysenteriae* serotype 1 and in shiga-toxin producing *E. coli* (STEC), with the *stx* gene located in a defective prophage and in lysogenic lambdoid prophages, respectively. However, recently multiple other shiga-toxin producing *Shigella* spp. were described that originated mainly from Haiti and the Dominican Republic. In these isolates, the *stx* gene is carried by the lysogenic prophage  $\phi$ POC-J13 and is easily transferred to other *Shigella* spp. and serotypes, posing a potential global threat [63, 64]. Besides the shiga-toxin, other toxins can be produced by *Shigella* spp. and EIEC. The chromosomally encoded enterotoxins ShET1 (*set* gene), pic (*pic* gene) and sigA (*sigA* gene) and the plasmid mediated ShET2 (*sen/ospD3* gene) and sepA (*sepA* gene), encoded on the pINV, can mediate the establishment of infection and causes the early stage watery diarrhea [48, 52, 65].

### Resistance

A mild to moderate shigellosis in otherwise healthy patients does not require treatment with antimicrobials, the infection will generally clear within a week. Antibiotic treatment is advised in more severe cases, immunocompromised patients or patients suffering from complications [20, 66]. However, the most important argument for antibiotic treatment of shigellosis is based on its epidemic potential. Prescription of antimicrobials as public health measurement, aiming to prevent extended fecal shedding, decreases the risk of secondary infections [66, 67]. The World Health Organization (WHO) advises to treat with ciprofloxacin, and in case of resistance, pivmecillinam and 3<sup>rd</sup> generation cephalosporins as alternative for children and azithromycin for adults [67]. Dutch guidelines advise to use co-trimoxazole if susceptibility has been proven, or ciprofloxacin and azithromycin as alternative [66, 68].

After the first use of antimicrobials in the 1940s, *Shigella* isolates almost immediately developed resistance to used antibiotics as sulphonamides, streptomycin, tetracycline and chloramphenicol. In 1956, *S. flexneri* was one of the first bacteria in which multi-drug resistance (MDR) was detected and described, although at that time the mechanism for this phenomenon was unknown [4].

Nowadays, antimicrobial resistance (AMR) of *Shigella* spp. is a major global public health concern, and 3<sup>rd</sup> generation cephalosporin resistance in *Enterobacteriaceae* in general and quinolone resistance in *Shigella* spp. specifically are on the global priority list of the WHO to guide research into alternative treatments [69].

AMR in *Shigella* spp. can be vertically inheritable and horizontally transferred via plasmid-encoded genes. Widely distributed are the resistance genes encoded on the MDR element

located in the *Shigella* resistance locus pathogenicity island (SRL-PAI) on the chromosomes of *S. dysenteriae*, *S. flexneri* and *S. sonnei* [3, 70-73]. The presence of the SRL-PAI infers resistance to  $\beta$ -lactam antibiotics, streptomycin, tetracycline and chloramphenicol. Multiple analyses have suggested it was acquired during multiple geographically associated occasions [3, 73]. Other chromosomally encoded resistance genes are located on the Tn7 transposon and the class II integron (Tn7/In2) that infer resistance to streptomycin and trimethoprim. Ciprofloxacin resistance is also chromosomally encoded, via substitutional point mutations in the *gyrA* and *parC* genes. For *S. sonnei*, South Asia is believed to be the reservoir for this ciprofloxacin resistant lineage, which expanded globally [71, 74]. Additionally to these chromosomally encoded resistance mechanisms, smaller and larger plasmids are present carrying multiple antimicrobial resistance genes, such as the spA plasmid, the pCERC1 plasmid and the pKSR100 plasmid [71, 72, 75, 76].

In *S. dysenteriae* and *S. sonnei*, the acquisition of elements that infer MDR was the onset of dominance for the most recent lineages and enabled clonal expansion and international spread [3, 76]. The success of these lineages is explained by a strong selective pressure, because the MDR lineages are able to circumvent antibiotic treatment and thus capable of causing prolonged shedding [71, 76]. In contrast, for *S. flexneri*, the acquirement of MDR did not result in global expansion, but merely independent acquisitions took place [73]. It was suggested that this difference could be explained by the transmission cycle of the organisms, as *S. flexneri* is capable of persistence in environmental circumstances, while *S. sonnei* and *S. dysenteriae* are directly transmitted from human to human only [73].

### Diagnostics

Since the discovery of *S. dysenteriae* by Dr. Shiga, both culturing techniques and agglutination with antisera are still widely used in routine diagnostics for identification of *Shigella spp.* and EIEC [4, 5]. Since these early days, multiple selective media were developed to facilitate selection of *Shigella spp.* from the other fecal flora and commercial monoclonal antisera for agglutination became available.

Despite their relatedness, *Shigella spp.* could always be distinguished from *E. coli* in general, based on their physiological and biochemical characteristics. Most *E. coli* (>80%) are motile, lysine decarboxylase (LDC) positive, form gas from D-glucose and are indole positive. On the contrary, *Shigella spp.* are by definition non-motile, LDC negative and never display the combination of gas and indole production. Acid formation from salicine and esculine hydrolysis were never described for *Shigella spp.*, while 40% and 35% of *E. coli* is positive for these features, respectively [9, 12, 77-81]. The distinction of *Shigella spp.* from the pathovar EIEC is more challenging, as EIEC can display either an *E. coli*-like profile, or a *Shigella*-like profile and all profiles in-between. Motility and the biochemical characteristics as LDC production, fermentation of salicin, esculin hydrolysis and the combined features

gas from D-glucose and indole production can aid in the differentiation of *Shigella spp.* from EIEC. Frequently used commercial systems as VITEK2 and Matrix-Assisted Laser-Desorption Ionization Time-of-Flight (MALDI-TOF) identification systems identify members of the pathovar *Shigella*-EIEC as either *E. coli* or *Shigella spp.* [82]. Multiple research groups developed custom tools for distinction of *Shigella spp.* and *E. coli* by MALDI-TOF, yet EIEC isolates were not considered in their development [83-85]. Supplementary to the physiological and biochemical characteristics, serotyping should be performed as EIEC is associated with specific *E. coli* O-serotypes. However, *E. coli* O-antisera should be used only in combination with *Shigella* antisera, because of the display of cross reactivity in antibody response [82].

Although culturing for detection and consecutive identification of *Shigella spp.* from fecal samples is widely adopted, it is insensitive, with a sensitivity of approximately 50% [16, 86]. Detection of EIEC by culture is even more challenging as no selective media are available and other *Enterobacteriaceae* are abundant in the fecal samples.

Since the last decade, molecular techniques primarily targeting the virulence gene *ipaH* are used for direct detection of *Shigella spp.* and EIEC in clinical samples [86, 87]. The *ipaH* gene is a multicopy gene on the pINV as well as on the chromosome, which is exclusively found in *Shigella spp.* and EIEC [46, 88, 89]. In the cascade of the virulence mechanisms, the *ipaH* gene is involved in the attenuation of the inflammatory response of the host [53]. The multicopy nature of the *ipaH* gene (4–10 copies) makes it a useful target for detection, even in case of plasmid loss or major deletions [89-92].

However, as *Shigella spp.* and EIEC both possess the *ipaH* gene, it is not suitable for distinction. This was the main driver for the search by multiple research groups for a molecular marker able to distinguish *Shigella spp.* from *E. coli* in general and EIEC in particular. For example, real-time PCRs were developed that target the *uidA*- and *lacY*-genes for distinction of EIEC from *Shigella spp.* [93, 94]. Another PCR was designed to separate the species of *Shigella*, using the *invC*-, *rfpB*-, *rfc*- and *wbgZ*-genes as genus and species-specific targets [95].

More recently, researchers have used sequences of whole genomes to develop molecular methods for identification of *Shigella spp.*, *E. coli* and EIEC based on k-mers or alignments of coding regions [49, 96, 97]. Other research groups classified *Shigella spp.* and EIEC in phylogenetic clades that better reflected the relatedness than the species designations, and proposed molecular diagnostics based upon this clade distribution [47, 50]. Although all these molecular methods showed above 95% accuracy using the originally selected set of isolates, they appeared not to be reliable when methods were repeated with another set of isolates [47, 97].

To summarize, despite all efforts to improve diagnostics, culture techniques for *Shigella* spp. and EIEC still have limited sensitivity and distinguishing the bacteria using molecular techniques is still challenging and unreliable.

### Epidemiology

The different species of *Shigella* have different geographical distributions. *S. dysenteriae* comprises 15 serotypes, of which serotype 1 is associated with outbreaks in displaced populations often due to war or natural disasters [3, 20, 98]. The incidence of *S. dysenteriae* has decreased dramatically over the last decades, in fact the last reported outbreaks date from the early 2000s [3, 98]. This decrease did not result from specific interventions, but is thought to have resulted from improved hygienic circumstances and sanitation, general improvements of nutrition, and introduction of measles vaccination preventing secondary dysentery infections [99]. Transmission of *S. dysenteriae* occurs probably through prolonged human carriage [65].

*S. boydii* is an uncommon isolated species that comprises 20 serotypes. It predominantly causes infections in patients from the Indian subcontinent, although some serotypes were related to travel to Central and South America [100, 101]. Both *S. dysenteriae* and *S. boydii* cause less than 10% of shigellosis cases globally, leaving the species *S. flexneri* and *S. sonnei* together account for more than 90% of the current global shigellosis burden [65, 102].

*S. flexneri* consists of 19 serotypes and has the ability to switch from serotype due to different serotype converting phages [103]. It is the species that is most attributable to shigellosis cases in low to middle-income countries. Transmission is thought to occur from environmental persistence in human contaminated water or food, as incidence lowers by clean water supply and good sanitation [73, 104, 105].

*S. sonnei* consists of one serotype encoded in an O-antigen cluster on the pINV, in contrast to other species of *Shigella* and *E. coli* that harbor their O-antigen clusters on the chromosome. It was hypothesized that *S. sonnei* derived their O-antigen cluster from *Plesiomonas shigelloides*, because their O-antigen clusters are almost identical [106, 107]. *S. sonnei* is the species most attributable to shigellosis in high-income countries, and with the economic development of a country, a shift in dominance from *S. flexneri* to *S. sonnei* has frequently been described [20, 108]. Multiple mechanisms were described to explain this shift. First, it was hypothesized that exposure to water of low quality, induces cross-immunity against *S. sonnei* due to the abundance of *P. shigelloides* that has similar O-antigens [109, 110]. Second, *S. sonnei* can use the common and widely present amoeba *Acanthamoeba castellanii* as protection against chlorination and other water sanitation processes. It is able to survive and experience enhanced growth in this amoeba, while *S. flexneri* kills it after phagocytosis [110, 111]. Third, *S. sonnei* is capable of easy acquirement of MDR through

horizontal gene transfer. This provides for advantages due to enhanced ability for infection, prolonged fecal shedding and the out-competition of other susceptible isolates [76, 110].

Populations most of risk of contracting a shigellosis infection are children, travelers and men who have sex with men (MSM) [16, 20, 66, 112]. Historically, shigellosis was thought to be transmitted via the fecal-oral route through human-to-human contact or via contaminated food or water. In the last decades, numerous high-resource countries, including the Netherlands, reported increased occurrence of shigellosis as sexually transmitted infection (STI) among MSM [17, 72, 113-116]. The species and serotypes predominantly detected in MSM populations are *S. sonnei* or *S. flexneri* serotypes 2a and 3a [71, 72, 114, 117]. The presence of shigellosis as STI in the MSM population is frequently associated with certain high-risk sexual practices, HIV positivity [113, 118, 119], and MDR [72, 75, 114, 115, 117, 118].

Although the ability of EIEC to cause diarrhea or food-related outbreaks was described, not much is known about its epidemiology, probably due to its problems with detection and identification [11, 19, 120-123].

### Public health threats and regulation in the Netherlands

Nowadays, the major public health threats regarding shigellosis are considered the national and international spread of multi resistant isolates or the recently described isolates that carry lysogenic prophages that encode the Shiga toxin, accommodated through travelling or MSM contact [17, 20, 64, 69, 114, 115, 119].

In the Netherlands, physicians and microbiological laboratories are obligated by law to notify local public health authorities for each confirmed case of shigellosis [124]. The criterion for confirmed case definitions in the Netherlands is the isolation of a *Shigella* spp. by culture [66]. In contrast, the European Union (EU), United States (US) and Australia recently amended their control guidelines for shigellosis with case definitions in which molecular detection of *Shigella* spp. is included in the criteria. In Australia, molecular detected shigellosis cases are notifiable, while in the EU and US, member countries and states determine their own notification guidelines [125-127].

Based on the notifications, Dutch local public health services contact every shigellosis case for source attribution, and collect epidemiological data. Subsequently, this data is collected from all regions at the centre for infectious disease control (CIb) at the National Institute for Public Health and the Environment (RIVM), to perform epidemiological surveillance for detection of national elevations or outbreaks.

In contrast to the employed public health regulations regarding the notifications and the subsequent control of infections with *Shigella* spp., infections with EIEC are not under any form of regulated control in the Netherlands.



## Objectives and outline of this thesis

Because of the challenging diagnostics of *Shigella spp.* and EIEC, laboratories struggle to fulfil Dutch public health regulations. First, culture confirmation is required for the case definition although detection of *Shigella spp.* by culture is insensitive. Molecular methods for detection from fecal samples are readily available, but are not able to distinguish the notifiable *Shigella spp.* from the pathotype EIEC that is not under control of public health regulations. Second, if an isolate is cultured, identification of *Shigella spp.* with the current available identification techniques is complex because of the resemblance to *E. coli*, and in particular, to the pathotype EIEC.

In the last decade, the relevance of used public health measures and the method of detection of shigellosis were questioned by public health authorities and microbiological laboratories in the Netherlands [87, 128]. The most important topics discussed were the significance of molecular detection and the current distinction regarding infections with *Shigella spp.* and EIEC in notification obligation, control regulations, and interventions. Although the two bacteria are genetically similar and are both able to cause a range of diarrheal diseases using the same mechanism, there is a gap of knowledge regarding incidence, epidemiology, clinical implications and impact on public health of infections with EIEC. Additionally, other countries reported genetic clustering of *Shigella spp.* based on MSM contact, travel history and resistance of isolates. Although a thorough epidemiological surveillance of *Shigella spp.* in the Netherlands is in place, that partially confirmed these patterns, laboratory surveillance is not part of control regulations for shigellosis.

A first objective of this thesis was to evaluate and optimize diagnostics for detection and differentiation of *Shigella spp.* and EIEC. Second, another objective was to fill knowledge gaps about incidence, epidemiology, clinical implications and impact on public health for EIEC infections. The outcomes were compared to outcomes of Dutch shigellosis cases to investigate if the current different approaches in control can be justified by evidence-based research. A third objective was to characterize circulating *Shigella spp.* and EIEC isolates in the Netherlands, in order to assess the population structure and molecular epidemiology.

In **part I**, the current situation regarding taxonomy and diagnostics in the Netherlands was determined. In **chapter 2**, the taxonomic status of *Shigella spp.* in relation to *E. coli* was evaluated using modern genomic taxonomic standards including all relevant type strains. Additionally, recommendations for reclassification were considered that are complying with the rules of nomenclature, yet are providing practicality for diagnostics, epidemiological surveillance and guidelines for infectious disease control. In **chapter 3**, the current used diagnostics for detection and identification of *Shigella spp.* and EIEC in the Netherlands was assessed. For this purpose, a collaborative laboratory trial was organized and results were

reported. In **part II**, the opportunities for the optimization of diagnostics for *Shigella spp.* and EIEC were explored. First, in **chapter 4**, a culture dependent algorithm was proposed and compared to a molecular algorithm. Subsequently, in **chapter 5**, three alternative approaches for identification of *Shigella spp.* and EIEC with MALDI-TOF were examined to assess their diagnostic accuracy, as commercially available databases are not able to distinguish them [129]. Because *Shigella spp.* and EIEC are genetically similar and are thought to cause the same symptoms and disease, the latter was studied in **part III** of this thesis. In this part, the results of a two-year cross-sectional study in the Netherlands were described, the Invasive Bacteria *E.coli-Shigella* Study (IBESS). During this study, *Shigella spp.* and EIEC isolates were collected in conjunction with epidemiological data of the infected patients. The aims of this study were to obtain more insights into the incidence, epidemiology, clinical implications and impact on public health of EIEC and *Shigella* infections. In **chapter 6**, the incidence, risks for infection, symptoms and severity of disease and socio-economic consequences of infections with EIEC and *Shigella spp.* were assessed and compared. Additionally, comparisons were made between culture confirmed cases and cases of which the bacteria were solely detected by molecular methods. In **chapter 7**, genome-wide association studies were applied to assess the presence of genetic markers in infecting isolates that can predict disease outcome for individual patients, irrespective of the identification as either *Shigella spp.* or EIEC. Such markers could potentially be used for development of molecular techniques that provide prioritization and optimization for public health guidelines. In **chapter 8**, the population structure of the isolates obtained during the IBESS-study was described. For this purpose, the isolates were phenotypically and genetically characterized and analyzed together with the obtained epidemiological data. Finally, in **chapter 9**, the results of all preceding chapters were summarized and discussed, and future perspectives and conclusions were presented.

The evidence provided in this thesis contributes to more understanding about the relatedness of *Shigella spp.* and EIEC, better tools for their distinction in the laboratory, and to practical suggestions for improvement of current public health guidelines for shigellosis.



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## Part I

Taxonomy and diagnostics of *Shigella spp.* and EIEC